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Human Tra2 Proteins Are Sequence-Specific Activators of Pre-mRNA Splicing

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Summary

The RNA-binding protein Tra2 is an important regulator of sex determination in *Drosophila*. Recently, two mammalian Tra2 homologs of unknown function have been described. Here, we show that human Tra2 proteins are present in HeLa cell nuclear extracts and that they bind efficiently and specifically to a previously characterized pre-mRNA splicing enhancer element. Indeed, both purified proteins bound preferentially to RNA sequences containing GAA repeats, characteristic of many enhancer elements. Neither Tra2 protein functioned in constitutive splicing in vitro, but both activated enhancer-dependent splicing in a sequence-specific manner and restored it after inhibition with competitor RNA. Our findings indicate that mammalian Tra2 proteins are sequence-specific splicing activators that likely participate in the control of cell-specific splicing patterns.

Introduction

Extraordinary examples of how alternative splicing can control eukaryotic gene expression are provided by the central steps in the regulatory cascade that determines sex-specific somatic differentiation in *Drosophila melanogaster* (for review, Burtis, 1993). In this cascade, female-specific expression of the *transformer* gene product Tra is accomplished by blockage of the strong default 3′ splice site by the female-specific Sex-lethal protein. The resulting utilization of the weaker female-specific 3′ splice site is required for expression of a functional Tra protein (Valcárcel et al., 1993). Tra in turn cooperates with the non–sex-specific protein Tra2 (the *transformer-2* gene product) to promote inclusion of the female-specific exon 4 into the *doublesex* (*dsx*) mRNA (Hoshijima et al., 1991; Tian and Maniatis, 1992). Exon 4 is associated with a weak 3′ splice site that is not used in male flies and contains a positive regulatory sequence, the so-called dsx enhancer, which consists of six 13-nucleotide repeats and a purine-rich element (Lynch and Maniatis, 1995). Tra and Tra2 bind cooperatively to the enhancer to which they recruit members of the SR protein family of general splicing factors (Inoue et al., 1992; Tian and Maniatis, 1993, 1994; Heinrichs and Baker, 1995; Lynch and Maniatis, 1996). SR proteins (for review, Manley and Tacke, 1996; Valcárcel and Green, 1996) and Tra2, which contain RNP-type RNA-binding domains (RBD), together with Tra are part of a superfamily of proteins characterized by domains rich in arginine/serine dipeptide repeats (RS domain) (for review, Fu, 1995). RS domains can participate in specific protein–protein interactions (Amrein et al., 1994; Kohtz et al., 1994; Xiao and Manley, 1997) and are generally phosphorylated in vivo (Gui et al., 1994; Colwill et al., 1996). Tra2 contains a single RBD flanked by two RS domains (Amrein et al., 1988; Goralski et al., 1989), whereas SR proteins contain one or two N-terminal RBDs and a C-terminal RS domain.

While the *Drosophila* sex-determination cascade illustrates that alternative splicing can result in strictly stage-specific expression patterns, splicing patterns of mammalian genes are often complex, and tissue- or stage-specific variations seem less pronounced. Perhaps because of this complexity, it has been difficult to identify mammalian factors involved in the tissue-specific control of single splicing events. A number of observations, however, suggest that SR proteins may participate in the maintenance and regulation of cell-specific splicing patterns. SR proteins are constitutive components of the splicing machinery that play important roles during spliceosome assembly by promoting splice site recognition (Crispino et al., 1994; Kohtz et al., 1994; Tarn and Steitz, 1994) and facilitating interactions between 5′ and 3′ splice site complexes (Wu and Maniatis, 1993; Tronchère et al., 1997). Individual SR proteins are able to complement splicing-deficient cytoplasmic S100 extracts, which lack SR proteins but contain other factors necessary for constitutive splicing (e.g., Ge et al., 1991; Krainer et al., 1991; Zahler et al., 1992). In addition, SR proteins can influence alternative splicing in a concentration-dependent manner, either when added to splicing-competent nuclear extracts (e.g., Ge et al., 1991; Krainer et al., 1991; Zahler et al., 1993) or when transiently overexpressed in vivo (e.g., Cáceres et al., 1994; Screaton et al., 1995; Wang and Manley, 1995). SR proteins have also been shown to participate in splicing activation of introns containing weak 5′ or 3′ splice sites by binding to purine-rich exonic splicing enhancers (ESE), frequently situated in downstream exons (Lavigueur et al., 1993; Sun et al., 1993; Staknis and Reed, 1994; Ramchatesingh et al., 1995; Yeakley et al., 1996). Conversely, high-affinity binding sites for the SR proteins ASF/SF2 (Tacke and Manley, 1995) and SRp40 (Tacke et al., 1997) have been demonstrated to function as ESEs in vitro. These and other studies (Fu, 1993; Chandler et al., 1997) also showed that SR proteins have distinct RNA binding specificities and display substrate specificity in vitro.

Unlike SR proteins, one of which, B52, has been shown to be required for development in *Drosophila* (Ring and Lis, 1994; Peng and Mount, 1995) and another, ASF/SF2, for viability of chicken DT40 cells (Wang et al., 1996), *Drosophila* Tra2 is apparently nonessential. Chromosomally female flies that are homozygous for a

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loss-of-function mutation in *transformer-2* are transformed into sterile phenotypical males but otherwise develop normally (Baker and Ridge, 1980). Tra2, together with Tra, regulates not only *dsx* pre-mRNA splicing but also sex-specific expression of the *fruitless* gene (Heinrichs et al., 1998), which controls sexual orientation and courtship behavior (Ryner et al., 1996). In addition, Tra2 is required for spermatogenesis in the male germ line, where it influences pre-mRNA processing in a Tra-independent manner (Mattox and Baker, 1991; Amrein et al., 1994; Hazelrigg and Tu, 1994; Madigan et al., 1996; Mattox et al., 1996). No functions of Tra2 outside sexual differentiation have so far been discovered in *Drosophila*. Recently, two human homologs of Tra2, Tra2α (Dauwalder et al., 1996) and Tra2β (Beil et al., 1997), have been identified. Using transgenic flies with nonfunctional Tra2, Dauwalder et al. (1996) demonstrated that human Tra2α is able to rescue Tra-dependent but not Tra-independent functions. The natural functions of mammalian Tra2 proteins are, however, unknown, as are their RNA binding specificities. Since the mechanisms of sexual differentiation are not conserved between mammals and flies, it is possible that human Tra2 proteins serve more general purposes than their *Drosophila* homolog.

Previously, we reported the creation of a purine-rich ESE, A3, which consists of three copies of a high-affinity ASF/SF2-binding site (Tacke and Manley, 1995). Here, we report the unexpected identification of a HeLa cell nuclear protein that binds specifically to A3 as one or both of the human Tra2 proteins. Using SELEX (Tuerk and Gold, 1990) and gel mobility-shift experiments, we find that Tra2α and Tra2β have indistinguishable RNA binding specificities and bind preferentially to oligo (GAA) sequences, such as are present in A3 and several natural splicing enhancers (e.g., Lavigueur et al., 1993; Xu et al., 1993; Yeakley et al., 1993). Splicing assays with HeLa S100 extracts, which lack Tra2 proteins, indicate that they are not required for constitutive splicing and cannot replace the essential splicing functions of SR proteins. However, both Tra2 proteins can activate A3-dependent splicing in a sequence-specific manner when added to limiting amounts of HeLa nuclear extracts and can restore enhancer-dependent splicing that had been inhibited by excess A3 competitor RNA. Together, our data indicate that Tra2α and Tra2β are sequence-specific activators of pre-mRNA splicing. The Tra2 proteins are the first mammalian proteins identified capable of functioning in this manner that are not also essential splicing factors, and they thus are likely to play important roles in splicing control.

Results

A 40 kDa SR Protein in HeLa Nuclear Extracts Binds Specifically to the A3 Enhancer

Using UV cross-linking assays, we showed previously that ASF/SF2 bound specifically to the A3 splicing enhancer in HeLa nuclear extracts (NE) but not to a similar purine-rich sequence, S3, that did not function as an enhancer. Furthermore, our data indicated that ASF/SF2 was necessary but not sufficient for activation of A3-dependent splicing in S100 extracts. In addition to ASF/SF2, a nuclear fraction, NF20-40, was also required (Tacke and Manley, 1995). To investigate further the interaction of the enhancer with SR proteins, we used an in vitro selection assay in which biotinylated A3 or S3 RNA was incubated in NE under splicing conditions, recovered with avidin agarose, and the bound proteins then analyzed by Western blots with the monoclonal antibody mAb104, which recognizes a phosphoepitope in RS domains (Roth et al., 1991). Surprisingly, a 40 kDa protein rather than ASF/SF2 was the predominant SR protein (i.e., a protein recognized by mAb104) selected by A3 (Figure 1, lane 1). Binding to A3 was specific, as no binding to S3 could be detected (Figure 1, lane 2), suggesting that this protein might play an important role in A3-dependent splicing.

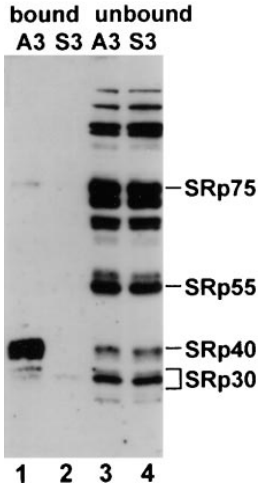

Figure 1. A 40 kDa SR Protein Binds Specifically to the A3 Enhancer

NE proteins bound to biotinylated A3 (lane 1) or S3 (lane 2) RNA were resolved by 9% SDS/PAGE and analyzed by Western blot with mAb104. Twenty-five percent of bound material is shown. Fractions (2%) of unbound material are shown in lanes 3 and 4.

To test whether the 40 kDa protein selected by A3 was the SR protein SRp40, we took advantage of polyclonal anti-SRp40 antibodies and the fact that SRp40 can be selected from NE by its genuine high-affinity binding site, B1 (Tacke et al., 1997). As shown in Figure 2, mAb104 generated signals of similar intensities with the 40 kDa protein selected by A3 (lane 2), authentic SRp40 selected by B1 (lane 4), and 60 ng of baculovirus-produced SRp40 (lane 6). Recognition by mAb104 was abolished in each case by treatment of the samples with alkaline phosphatase (CIP) before Western analysis (Figure 2, lanes 3, 5, and 7). In contrast, anti-SRp40 antibodies recognized the B1-selected protein (Figure 2, lane 10) and baculovirus-produced SRp40 (Figure 2, lane 12) but not the A3-selected protein (Figure 2, lane 8). Identical results were obtained with the CIP-treated samples (Figure 2, lanes 9, 11, and 13). Note that mAb104 and anti-SRp40 antibodies also recognized an unidentified SRp30 protein selected by B1 (Figure 2, lanes 4, 10 and 11), as previously reported (Tacke et al., 1997). Our results strongly suggest that the 40 kDa protein binding to A3 is not SRp40. This conclusion was corroborated by selection experiments with the nuclear

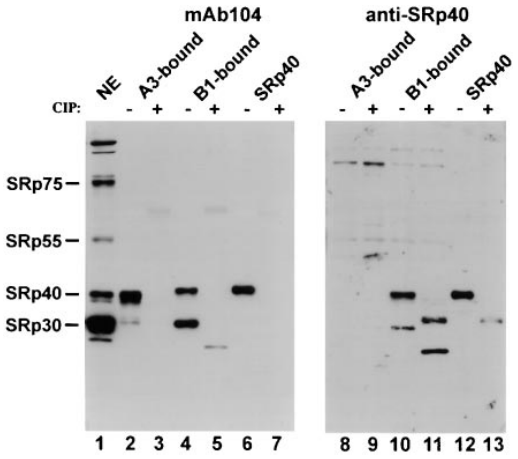

Figure 2. The A3-Binding 40 kDa Protein Is Not SRp40

Shown are Western blots with mAb104 (left) and anti-SRp40 antibodies (right) of NE proteins bound to biotinylated A3 (lanes 2, 3, 8, and 9) or B1 RNA (lanes 4, 5, 10, and 11) and of 60 ng purified SRp40 (lanes 6, 7, 12, and 13). Samples were either untreated (−) or treated with CIP (+), which abolished recognition by mAb104. Lane 1, fraction of input material. Note that B1 binds an unidentified 30 kDa SR protein in addition to SRp40.

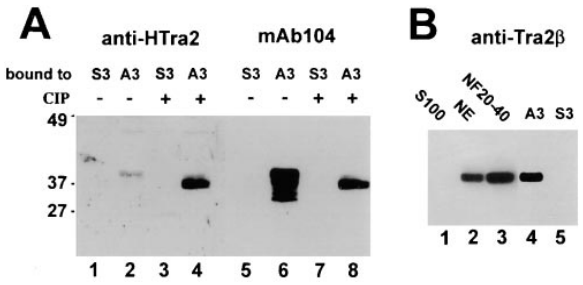

Figure 3. A Human Tra2 Protein Is Recognized by mAb104 and Binds Specifically to A3

(A) NE proteins bound to biotinylated A3 or S3 RNA were analyzed by Western blot with anti-Tra2 antibodies (lanes 1–4) or mAb104 (lanes 5–8) before (−) or after (+) treatment with CIP. As a result of partial dephosphorylation in this experiment, CIP treatment did not abolish recognition by mAb104 but enhanced mobility of the recognized proteins.

(B) Western analysis with anti-Tra2β-antibodies of S100 (lane 1), NE (lane 2), NF20-40 (lane 3), and NE proteins selected by A3 (lane 4) or S3 (lane 5).

fraction NF20-40. A 40 kDa protein was selected by A3 but not by B1, indicating that NF20-40 contains the A3-binding protein but not SRp40 (data not shown).

The A3-Binding Protein Recognized by mAb104 Is a Tra2 Protein

What is the identity of the A3-binding protein? Recent cloning of two mammalian homologs of the *Drosophila* splicing regulator Tra2, Tra2α (Dauwalder et al., 1996) and Tra2β (Matsuo et al., 1995; Segade et al., 1996; Beil et al., 1997), revealed that both proteins are highly homologous RS domain proteins with extended stretches of RS dipeptide repeats, especially in their N-terminal RS domains. Moreover, SRp40, Tra2α, and Tra2β all have very similar predicted molecular weights and amounts of R and S residues. We therefore reasoned that phosphorylated human Tra2 proteins, if present in HeLa cells, might be recognized by mAb104 and display migration properties similar to SRp40 in SDS/polyacrylamide gels. To test whether phosphorylated Tra2 proteins are recognized by mAb104, His-tagged versions of both proteins (HTra2α and HTra2β) were expressed in and purified from *Escherichia coli*. Indeed, both HTra2α and HTra2β, which were not recognized by mAb104 in their unphosphorylated states, reacted strongly with the antibody either after incubation under splicing conditions in NE or S100 or following phosphorylation by the SR protein kinase Clk/Sty (data not shown).

To determine whether the A3-binding protein recognized by mAb104 was in fact a Tra2 protein, we raised polyclonal antibodies against HTra2α. The resulting antibodies recognized both HTra2α and HTra2β, owing to the high degree of sequence identity between the two proteins, but not recombinant SRp40 or proteins present in a standard SR protein preparation (data not shown). As shown in Figure 3A, a 40 kDa protein selected from NE specifically by A3 (lane 2) but not S3 (lane 1) was detected with the antibodies. This protein comigrated with the A3-binding protein identified by mAb104 (lane 6). CIP treatment of the samples enhanced recognition by the anti-HTra2 antibodies (lane 4) and, in addition, resulted in equivalent mobility-shifts of the proteins recognized by the two different antibodies (compare lanes 4 and 8), confirming that the proteins are indeed identical. We conclude that one or both of the human Tra2 proteins bind specifically to the A3 enhancer.

We next wished to determine the distribution of Tra2 in various subcellular fractions, as this could provide some insight into the possible function of the proteins. However, because of the low affinity of the anti-Tra2 antibodies to phosphorylated Tra2 proteins, a highly specific anti-Tra2β antibody (see Experimental Procedures) was used to address this question. As shown in Figure 3B, Tra2β was not detected in S100 (lane 1) but was readily detected in both NE (lane 2) and NF20-40 (lane 3). In addition, Tra2β was selected by A3 but not S3 from NE (lanes 4 and 5) or NF20-40 (data not shown). These results confirm that Tra2β can interact specifically with a splicing enhancer, and its presence in NF20-40 but not S100 is consistent with a possible role in the function of such elements.

RNA Binding Specificities of Human Tra2α and Tra2β

How do the Tra2 proteins bind to the A3 enhancer? One possibility is that they fortuitously recognize an RNA sequence similar to the ASF/SF2 recognition motif present in A3. Alternatively, they could be recruited to the enhancer not by direct RNA binding but by interaction with other proteins (e.g., ASF/SF2), which would be consistent with their preferential detection by biotin selection as opposed to UV cross-linking. To distinguish between these possibilities, we decided to ascertain the RNA binding specificities of both proteins and to determine whether these would be consistent with the idea

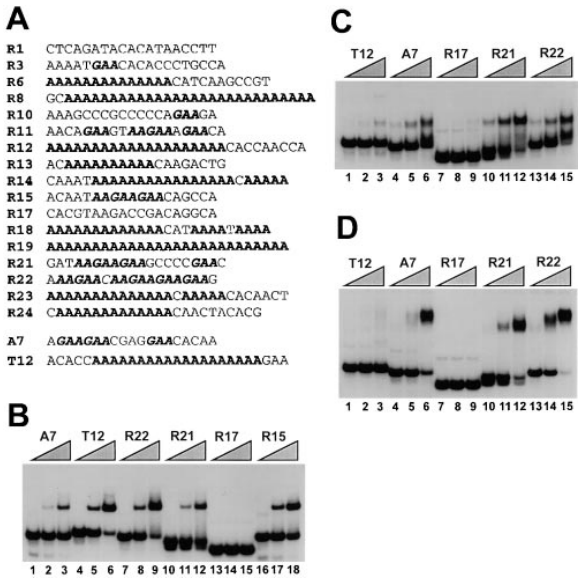

Figure 4. RNA Binding Specificities of Tra2α and Tra2β

(A) Sequences selected by Tra2β after five rounds of SELEX (R1-R24). A7 and T12 represent sequences selected by ASF/SF2 and Tra2α, respectively. Poly(A) sequences are in boldface; GAA motifs are in italicized boldface.

(B) Gel mobility-shift assays of the indicated radiolabeled sequences were performed with increasing concentrations (3, 15, and 75 nM) of HTra2α.

(C) and (D) Gel mobility-shift assays with 1.2, 6, and 30 nM of baculovirus-produced Tra2α (C) or with 2, 10, and 50 nM of bacterially produced GST-Tra2β (D).

that the proteins bind directly to A3 and perhaps other purine-rich splicing enhancers. To this end, we first performed SELEX with HTra2α and HTra2β that had been phosphorylated in vitro by Clk/Sty (Tacke et al., 1997). Selected sequences were inspected after five cycles of selection and amplification. Unexpectedly, all of 20 sequences selected by HTra2α contained extended stretches of poly(A). In one case, the entire selected sequence consisted of a continuous poly(A) sequence of 24 adenosine residues (data not shown). Sequences selected by HTra2β showed greater variety (Figure 4A). Although many of them also contained stretches of poly(A), others contained motifs of oligo(GAA), which, remarkably, are also found in A3 and a number of naturally occurring splicing enhancers (e.g., Lavigueur et al., 1993; Xu et al., 1993; Yeakley et al., 1993).

To test the significance of these sequences as binding sites for HTra2α and HTra2β, we performed gel mobility-shift experiments with both proteins. Since no significant differences were found between the binding specificities of the two proteins (data not shown), the findings are exemplified in Figure 4B by an experiment with HTra2α. Four oligo(GAA)-containing sequences, three of which were selected by HTra2β (R15, R21, and R22) and one by a truncated version of ASF/SF2 (A7; Tacke and Manley, 1995); a poly(A)-containing sequence selected by HTra2α (T12); and an HTra2β-selected sequence containing neither poly(A) nor oligo(GAA) (R17) were radiolabeled and incubated with 3, 15, or 75 nM of phosphorylated, repurified HTra2α. Efficient binding was observed with all sequences containing either poly(A) or oligo(GAA) but not with R17. Notably, binding was also observed with the sequence A7, containing the ASF/SF2-binding site AGAAGAAC, three copies of which constitute the A3 enhancer.

Unlike oligo(GAA), poly(A) appears to be unable to function as a splicing enhancer (Tanaka et al., 1994), and attempts to recover Tra2 proteins from NE with biotinylated poly(A) RNA were unsuccessful (data not shown). This raises the possibility that authentic Tra2 proteins might not recognize poly(A). To obtain a recombinant Tra2 protein that resembled the authentic protein as closely as possible, untagged Tra2α (Tra2α) was expressed in baculovirus-infected insect cells and purified by classical biochemical methods (see Experimental Procedures). As shown in Figure 4C, this protein bound efficiently to oligo(GAA)-containing sequences (A7, R21, R22) but poorly to the poly(A)-containing sequence T12. We also attempted to purify untagged Tra2β from baculovirus-infected insect cells, but for technical reasons only a fraction enriched in Tra2β could be prepared (see Experimental Procedures). Therefore, a fusion protein of glutathione-S transferase and Tra2β was expressed in and purified from *E. coli*, phosphorylated in HeLa S100 extracts, and repurified (GST-Tra2β). Similar to Tra2α, GST-Tra2β bound efficiently only to the oligo(GAA)-containing sequences (A7, R21, and R22) (Figure 4D). Hence, we conclude that Tra2α and Tra2β have very similar RNA binding specificities, binding preferentially to oligo(GAA)-containing sequences. We do not know the reason for selection of poly(A) sequences by SELEX but suspect that it may reflect the presence of the His-tag on the recombinant proteins used in this assay.

Tra2α and Tra2β Stimulate A3-Dependent Splicing but Cannot Substitute for the Essential Splicing Function of SR Proteins

Taken together, our results provide evidence that both Tra2α and Tra2β can bind directly to A3 and possibly other purine-rich splicing enhancers. What is the functional significance of this, and how, if at all, do Tra2 proteins function in splicing? An important feature of SR proteins is that any one of them is sufficient to complement splicing-deficient S100 extracts for splicing of at least some substrates containing consensus or near-consensus splice sites. Based largely on this feature, SR proteins have been termed general, essential, or constitutive splicing factors and are thought to be redundant in this assay. We first sought to determine whether Tra2 proteins might have similar functions. Significantly, both Tra2α (Figure 5A, lanes 4 and 5) and GST-Tra2β (data not shown) were unable to activate splicing of a β-globin pre-mRNA in S100, although efficient splicing was observed with ASF/SF2 (Figure 5A, lanes 2 and 3). Identical results were obtained with PiP7A RNA (Kohtz et al., 1994) as a substrate (data not shown). We next tested whether Tra2α or Tra2β was able to complement S100 for splicing of a pre-mRNA containing the A3 enhancer. As a splicing substrate, we used an IgM-based pre-mRNA with a suboptimal pyrimidine tract (Watakabe et al., 1993) containing the A3 enhancer downstream of the 3′ splice site (M-A3; see Experimental Procedures).

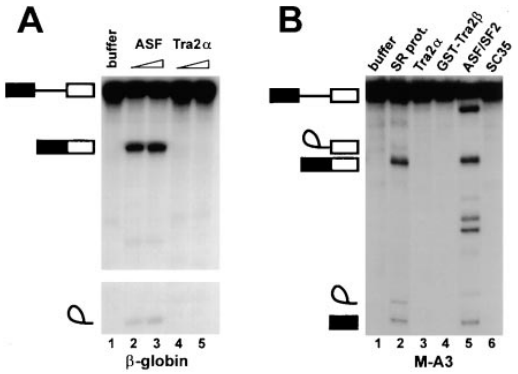

Figure 5. Tra2 Proteins Cannot Substitute for Essential Splicing Functions of SR Proteins

In vitro splicing of β-globin (A) and M-A3 (B) pre-mRNAs in S100 supplemented with no additional protein (lane 1) or purified proteins as indicated.

(A) Efficient splicing of β-globin with 60 (lane 2) or 120 (lane 3) nM ASF/SF2; no splicing with 60 (lane 4) or 120 (lane 5) nM Tra2α. (B) Efficient splicing of M-A3 with 1 μg/assay of purified SR proteins (lane 2) or 600 nM ASF/SF2 (lane 5); no splicing with 360 nM Tra2α (lane 3), 360 nM phosphorylated GST-Tra2β (lane 4), or 600 nM SC35 (lane 6).

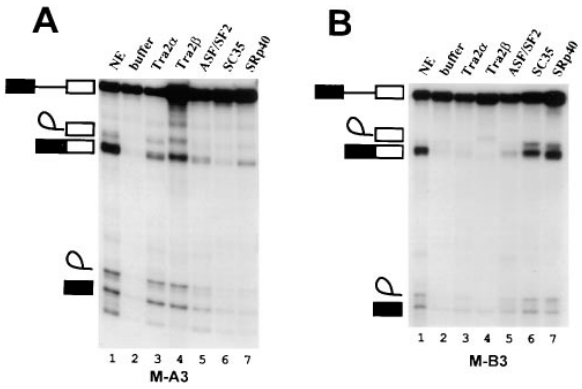

Figure 6. Tra2 Proteins Specifically Stimulate A3-Dependent Splicing

In vitro splicing of M-A3 (A) or M-B3 (B) in NE (lane 1) or limiting amounts of NE supplemented with no protein (lane 2), Tra2α (lane 3), a fraction enriched in Tra2β (lane 4), or individual SR proteins as indicated (lanes 5–7). Recombinant purified proteins were used at 120 nM in all reactions.

As shown in Figure 5B, SR proteins purified from HeLa cells (lane 2) and ASF/SF2 (lane 5) were active in this assay, although high concentrations (1 μg/assay of SR proteins or 600 nM ASF/SF2) were required for efficient splicing. In addition, ASF/SF2 produced unidentified bands, probably reflecting aberrant splice site and/or branch point usage (lane 5). In contrast to ASF/SF2, Tra2α (lane 3) and GST-Tra2β (lane 4) were inactive. As expected, SC35, which binds the A3 enhancer poorly (Tacke and Manley, 1995), was also inactive (lane 6). We conclude that Tra2 proteins cannot carry out essential splicing functions executed by SR proteins, neither with consensus-type substrates nor with a pre-mRNA containing an enhancer with high-affinity binding sites for Tra2 proteins.

The above results indicate that the human Tra2 homologs, in the absence of SR proteins, are unable to activate enhancer-dependent splicing. However, these findings do not address the possibility that one or both of these proteins might nonetheless play an important role in enhancer-dependent splicing under more physiological conditions. Therefore, we employed an assay in which limiting amounts of NE (e.g., Yeakley et al., 1996) that promoted very little if any splicing of M-A3 were supplemented with Tra2β, purified Tra2α, or different SR proteins. Most importantly, both Tra2α and Tra2β significantly enhanced M-A3 splicing (Figure 6A, lanes 3 and 4). The SR proteins tested were also active in this assay, but stimulation was reduced, especially with SC35 (Figure 6A, lanes 5–7). Stimulation by SR proteins may partly reflect general splicing functions that are independent of the enhancer. However, Tra2 proteins are unable to carry out such functions (see above). Thus, stimulation of M-A3 splicing by Tra2 proteins likely depends upon their ability to bind to the A3 enhancer. A prediction from this is that a related substrate, M-B3, containing the SRp40-dependent B3 enhancer (Tacke et al., 1997), which does not bind the Tra2 proteins, in place of A3 will not respond to Tra2. Indeed, neither Tra2α nor Tra2β activated splicing of M-B3 in limiting amounts of NE (Figure 6B, lanes 3 and 4). In contrast, stimulation was again observed with the SR proteins, although in this case it was very slight with ASF/SF2 (Figure 6B, lanes 5–7; activation likely reflects cooperation with SRp40 present in NE). Hence, sequence-specific binding of Tra2 to A3 likely plays a role in the activation of A3-dependent splicing in NE.

To extend the above results, we performed competition assays in NE, using as competitors the A3, B3, and S3 sequences. As shown in Figure 7A, 5-fold excess of A3 decreased the splicing efficiency of M-A3 in NE dramatically (lane 2), while 15-fold excess of B3 had no effect (lane 5). S3, which does not function as an enhancer (Tacke and Manley, 1995), also had no effect as a competitor (Figure 7A, lanes 6 and 7). Most importantly, inhibition of A3-dependent splicing by A3 competitor

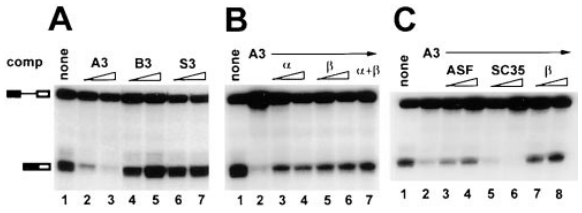

Figure 7. Tra2 Proteins Restore M-A3 Splicing Inhibited by Excess A3

(A) Splicing of M-A3 in NE is inhibited by 5-fold (lanes 2, 4, and 6) or 15-fold (lanes 3, 5, and 7) molar excess of A3, but not B3 or S3. Lane 1, no competitor.

(B) Sixty or 120 nM Tra2α (lanes 3 and 4), 60 or 120 nM phosphorylated GST-Tra2β (lanes 5 and 6), or a combination (60 nM each) of both (lane 7) restore M-A3 splicing inhibited by a 10-fold excess of A3.

(C) Sixty or 240 nM ASF/SF2 (lanes 3 and 4), but not SC35 (lanes 5 and 6), partially restores splicing inhibited by 10-fold excess of A3 but less effectively than 30 or 120 nM GST-Tra2β (β) (lanes 7 and 8). Only the splicing substrates and mRNA products are shown.

RNA could be relieved by Tra2α (Figure 7B, lanes 3 and 4), GST-Tra2β (Figure 7B, lanes 5 and 6; Figure 7C, lanes 7 and 8), or a combination of both (Figure 7B, lane 7). Depending on the experiment, 30–120 nM of GST-Tra2β was sufficient to restore 50%–100% of the splicing efficiency obtained in the absence of competitor RNA. ASF/SF2 was able to partially restore M-A3 splicing inhibited by A3 (Figure 7C, lanes 3 and 4) but was significantly less effective than the Tra2 proteins. For example, splicing efficiencies were similar with 240 nM ASF/SF2 and 30 nM GST-Tra2β (Figure 7C, compare lanes 4 and 7). SC35 did not restore splicing and in fact further decreased splicing efficiency at higher concentrations (Figure 7C, lanes 5 and 6). Together, our results demonstrate that Tra2 proteins can specifically activate A3-dependent splicing, likely reflecting their ability to bind the enhancer with high affinity.

Discussion

In this paper, we have shown that the human homologs of the *Drosophila* Tra2 protein are present in HeLa nuclear extract and, unexpectedly, bind efficiently to a splicing enhancer consisting of high-affinity binding sites for the SR protein ASF/SF2. Both Tra2 proteins bound tightly and specifically to RNA sequences containing GAA repeats, which are similar both to the ASF/SF2 consensus recognition motif and to sequences found in a number of naturally occurring splicing enhancers. We have provided evidence that the Tra2 proteins are not required for constitutive splicing but instead play an important role in enhancer-mediated activation of splicing. Thus, the Tra2 proteins on the one hand appear to play a more restricted role in splicing than do the structurally related SR proteins, while on the other hand they display properties indicative of a critical function in splicing control.

RNA Binding Specificities of Tra2 Proteins

Our data suggest that human Tra2 proteins and the SR protein ASF/SF2 have overlapping RNA binding specificities. Both Tra2α and GST-Tra2β recognized the sequence A7, which was previously obtained by SELEX with a truncated version of ASF/SF2 lacking its RS domain. A7 contains a perfect match of the ASF/SF2 consensus octamer AGAAGAAC and constitutes a high-affinity binding site for authentic ASF/SF2 (Tacke and Manley, 1995). Based on gel-shift assays with A7, we estimate that ASF/SF2 and Tra2α bind this sequence with similar affinities (i.e., with apparent K_D values of approximately 50 and 30 nM, respectively). Surprisingly, binding of Tra2 proteins present in NE to biotinylated A3 could be readily detected, while binding of ASF/SF2 was weak. In contrast, UV cross-linking assays using A3 with radioactively labeled G nucleotides initially suggested that ASF/SF2 bound A3 more efficiently than other nuclear proteins, in particular more efficiently than a 40 kDa protein that likely represented a Tra2 protein but was previously considered to be SRp40 (Tacke and Manley, 1995). This discrepancy may reflect the different properties of the two assays, different cross-linking efficiencies of the two proteins, and/or different binding properties of the proteins. In fact, changing the labeled base from G to A strongly increased the cross-linking efficiency of Tra2 proteins compared to ASF/SF2 (R. T. and J. L. M., unpublished data), confirming the high-affinity of Tra2 for A3 and also indicating that the proteins recognize the A3 sequence in different ways. The latter may be expected, as Tra2 and ASF/SF2 differ in the number of RNA-binding domains they contain (one and two, respectively) and do not share significant homology with each other in the RNA-binding regions. This makes it all the more remarkable that these two splicing factors with related but clearly distinct functions and properties recognize very similar RNA sequences.

The physiological significance of the related RNA binding specificities of ASF/SF2 and Tra2 proteins remains to be determined. However, a previous analysis of the dsx enhancer is consistent with the possibility that ASF/SF2 and *Drosophila* Tra2 also possess overlapping RNA binding specificities. Both proteins were shown to bind the dsx enhancer with 4- to 5-fold lower efficiencies when an 18-base purine-rich element (PRE) was deleted (Lynch and Maniatis, 1995). The two proteins also bound the isolated PRE, with *Drosophila* Tra2 showing the highest specificity for this sequence, despite the fact that the PRE lacks a GAA triplet. Whether this reflects the fact that the PRE is a low-affinity Tra2-binding site or is indicative of a slight difference in binding specificity between *Drosophila* and human Tra2 proteins is not known. The human and *Drosophila* Tra2 RBDs are 54% identical.

Tra2 Proteins as Mammalian Splicing Regulators

Unlike SR proteins, Tra2 proteins appear not to function as constitutive splicing factors despite their overall structural similarities to SR proteins. SR proteins possess distinct RNA binding specificities (e.g., Heinrichs and Baker, 1995; Tacke and Manley, 1995; Tacke et al., 1997), and their RS domains function redundantly in certain assays in vitro (Chandler et al., 1997) and are entirely interchangeable in vivo (J. Wang and J. L. M., submitted). This suggests that neither the RNA binding specificity nor the nature of the RS domain is a decisive determinant for constitutive splicing activity. In fact, viability of DT40 cells genetically depleted of ASF/SF2 can be rescued by chimeric ASF/SF2 proteins in which the genuine RS domain was replaced by either the N- or C-terminal RS domain of Tra2α (J. Wang and J. L. M., submitted), indicating that both Tra2α RS domains can perform the essential functions of the ASF/SF2 RS domain. It is, however, possible that the overall domain organization of Tra2 proteins, which differs from that of SR proteins, is incompatible with a function as a constitutive splicing factor, perhaps by preventing crucial protein–protein interactions with other essential splicing factors, such as the U1 snRNP 70 kDa protein or U2AF (Wu and Maniatis, 1993; Kohtz et al., 1994; Zuo and Maniatis, 1996). In addition, Tra2 and SR proteins may differ in the way they position themselves on the RNA. In this context, it is noteworthy that despite their differences in RNA binding specificities, SR proteins possess common motifs in their RNA-binding domains, which are characteristic of the family (Birney et al., 1993) but are not shared with Tra2 proteins.

Unlike M-A3 pre-mRNA, which could be spliced in S100 with purified SR proteins or ASF/SF2 alone, splicing of another A3-enhancer substrate, GN-A3, required in addition the nuclear fraction NF20-40 (Tacke and Manley, 1995). It is likely that part of the activity of NF20-40 is due to the presence of Tra2 proteins in this fraction. However, NF20-40 could not be replaced by Tra2 proteins and, in addition, is not essential for M-A3 splicing. Additional factors in NF20-40 are therefore likely required by specific features of the GN-A3 substrate other than the enhancer sequence itself. Notable among the differences between the GN-A3 and M-A3-substrates are the closer proximity of the enhancer to the 3′ splice site in M-A3 and the fact that M-A3 contains a weak 3′ splice site, while GN-A3 contains a weak 5′ splice site.

Previous studies showed that SR proteins present in nuclear extracts bind to purine-rich ESEs (e.g., Lavigueur et al., 1993; Sun et al., 1993; Ramchatesingh et al., 1995) and are present in enhancer complexes (Staknis and Reed, 1994). Identification of SR proteins has mainly relied on their size and recognition by mAb104. Our data indicate that it may be difficult to distinguish between human Tra2 proteins and SRp40 with mAb104. Recently, Yeakley et al. (1996) reported specific binding of a 40 kDa nuclear extract protein to an enhancer element consisting of an oligo(GAA)-containing sequence. The authors identified the protein as SRp40 on the basis of its reactivity with mAb104, but they were unable to detect binding of SRp40 when using purified SR proteins. Based on these and additional findings, they concluded that SRp40 was recruited to the enhancer by a cofactor or complex present in NE. In the light of our data, it seems likely that the 40 kDa SR protein was one or both of the human Tra2 proteins rather than SRp40 and that it bound the oligo(GAA) element directly. This conclusion is significant because it questions the view that RBD-containing RS domain proteins can be recruited to splicing complexes independent of their RNA binding activity. A 40 kDa protein in HeLa NE shown to be cross-linked by UV to an oligo (GAA)-containing ESE in the cardiac troponin T pre-mRNA probably also represents a human Tra2 protein (Ramchatesingh et al., 1995).

Several studies have shown that SR proteins, including ASF/SF2 and SRp40, can participate in the activation of ESE-dependent splicing in vitro (Sun et al., 1993; Ramchatesingh et al., 1995; Tacke and Manley, 1995; Yeakley et al., 1996; Tacke et al., 1997). Although in most instances nuclear extracts containing Tra2 were used, in one case SR proteins were shown to activate ESE-dependent splicing in S100 extracts (Ramchatesingh et al., 1995). This is consistent with our results and suggests that human Tra2 proteins need not be absolutely required for ESE-dependent splicing. How critical then is the role of Tra2 proteins? In our experiments, high concentrations of purified SR proteins or ASF/SF2 (600 nM) were required to activate A3-dependent splicing in S100. Such high concentrations are unlikely to reflect physiological conditions, as the concentrations of individual SR proteins in our HeLa NE are less than 50 nM (R. T. and J. L. M., unpublished data). Under more physiological conditions, as in our assays using NE, both human Tra2 proteins activated splicing in a sequence-specific manner and were significantly more effective than ASF/SF2. Thus, in the case of natural enhancers that appear to contain high-affinity Tra2 binding sites (e.g., Lavigueur et al., 1993; Xu et al., 1993; Yeakley et al., 1993; Ryan and Cooper, 1996; Elrick et al., 1998), the Tra2 proteins likely play important roles in the control of enhancer-dependent splicing patterns in vivo, perhaps by recruiting SR proteins or other factors to the enhancer. Cooperative interactions between *Drosophila* Tra2 and SR proteins have been documented (Lynch and Maniatis, 1996), and there is also evidence that human Tra2 proteins can interact with SR proteins (Beil et al., 1997; Shin et al., unpublished data).

What are the specific functions of the two Tra2 proteins in vivo? Our data have not revealed functional differences between Tra2α and Tra2β. Their RBDs are 85% identical, consistent with our finding that their RNA binding specificities are indistinguishable. The proteins differ mainly in the N-terminal 49 amino acids and in the position of a polyglycine stretch within their C-terminal RS domains, which otherwise are highly homologous. One possibility consistent with our data is that the two proteins are functionally redundant in vivo. Alternatively, the small differences in primary structure may account for potential differences in protein–protein interactions, including with constitutive splicing factors such as SR proteins or with cell-specific factors yet to be discovered. An important aspect of the function of the *Drosophila* Tra protein in the regulation of dsx splicing appears to be its ability to influence the RNA binding properties of Tra2 through cooperative interaction. Whereas Tra2 binds preferentially to the purine-rich element of the dsx enhancer in the absence of Tra, it can also bind to the dsx repeats in the presence of Tra (Lynch and Maniatis, 1995, 1996). It is tempting to postulate a scenario in which cell-specific factors alter the RNA binding properties of human Tra2 proteins analogous to the Tra/Tra2 cooperation in *Drosophila*. It is also noteworthy that Tra2β was initially identified as a factor rapidly induced during reoxygenation of astrocytes after hypoxia (Matsuo et al., 1995) and subsequently shown to display different mRNA expression levels in various mouse tissues (Segade et al., 1996). These examples raise the possibility that at least in some cases mammalian Tra2 proteins themselves might act as stage-specific regulators of pre-mRNA splicing. As in *Drosophila*, in vivo genetic studies may ultimately be required to elucidate the precise roles of mammalian Tra2 proteins in the control of cell- and stage-specific splicing patterns.

Experimental Procedures

Constructs

The cDNA for the coding region of human Tra2α was obtained as an NdeI-BamHI fragment by reverse transcription/PCR of total HeLa cell RNA. To express HTra2α in *E. coli* the fragment was inserted into pET14b (Novagen). After transfer to Bluescript KSII (Stratagene), the cDNA was inserted as an XbaI-BamHI fragment into the baculovirus expression vector pVL1392 (Pharmingen). To express HTra2αΔRSN (see below) in *E. coli* the Tra2α cDNA lacking coding sequences upstream of the single EcoRII site was subcloned into pET14b. The full-length cDNA of human Tra2β was isolated from a human brain cDNA library using the previously obtained rat cDNA (Matsuo et al., 1995) as a probe. The coding region was cloned as an NdeI-BamHI PCR fragment into pET14b for the bacterial expression of HTra2β and eventually transferred to pFASTBAC1 for the

generation of a recombinant baculovirus using the BAC-TO-BAC expression system (GIBCO BRL). To express GST-Tra2β the coding region was inserted blunt into the BamHI site of pGEX-2T (Pharmacia).

Purification of Recombinant Proteins

His-tagged recombinant proteins were expressed in BL21, purified under denaturing conditions by Ni^{2+} agarose chromatography, and renatured by dialysis (Tacke and Manley, 1995). GST-tagged proteins were expressed in JM101 and purified under nondenaturing conditions by glutathione agarose chromatography as described (Xiao and Manley, 1997). SR proteins from HeLa cells and recombinant SR proteins expressed in baculovirus-infected Sf9 cells were purified as previously described (Tacke et al., 1997). Tra2α was purified from baculovirus-infected Sf9 or High Five insect cells (Invitrogen). After lysis of the cells by sonication in 10 volumes of isolation buffer (Zahler et al., 1992), the homogenate was cleared by centrifugation at 10,000 × g for 30 min, ammonium sulfate was added to 40% saturation, and precipitation was allowed to proceed for 30–60 min. The soluble material was recovered by centrifugation at 10,000 × g, and up to 15 mg was loaded onto a 1 ml Phenyl Superose column (TosoHaas) using the FPLC system (Pharmacia). Bound material was eluted with a linear salt gradient of 1.5–0 M ammonium sulfate in buffer A (20 mM HEPES [pH 7.9], 5 mM KF, 1 mM EDTA, 0.5 mM DTT). Fractions containing Tra2α were pooled, and the ammonium sulfate concentration was adjusted to 200 mM by dialysis. At that stage of the purification, Tra2α was at least 50% pure when purified from High Five cells. To obtain Tra2α of higher than 90% purity, the protein was further purified by hydroxylapatite chromatography using a 0–600 mM potassium phosphate gradient in buffer A (without HEPES) for elution and finally dialyzed against modified buffer D (Ge et al., 1991) containing 50 mM ammonium sulfate. Phenyl Superose fractions enriched in Tra2β were obtained essentially as described for Tra2α and used for functional assays after dialysis against modified buffer D. However, because Tra2β displayed lower expression levels in insect cells than Tra2α and was less soluble in fractions containing ammonium sulfate at 40% saturation, purity was below 10% after Phenyl Superose chromatography.

Phosphorylation of Recombinant Proteins

Two hundred micrograms of His-tagged proteins was incubated with 20 μg of GST-Clk as described previously (Colwill et al., 1996) and repurified by Ni^{2+} agarose chromatography as described above. GST-Tra2β was phosphorylated in S100 under standard splicing conditions for 30 min (Tacke et al., 1997) and repurified as described above.

Antibodies

Affinity-purified anti-SRp40 antibodies have been described previously (Tacke et al., 1997). Polyclonal antibodies that recognize both human Tra2 proteins were raised against a truncated version (amino acids 56-282) of Tra2α lacking part of the N-terminal RS domain (HTra2αΔRSN). Affinity purification of the antiserum with the protein coupled to cyanogen-activated Sepharose (Pharmacia) beads was performed using standard procedures. Purified antibodies were eventually eluted with 100 mM glycine (pH 2.2). Eluates were immediately neutralized with 0.05 volumes of 1 M Tris HCl (pH 9.0) and supplemented with 0.1 μg/μl bovine serum albumin for long-term storage at −70 °C. Polyclonal antibodies specific for Tra2β were raised against and affinity-purified with a synthetic peptide comprising amino acids 2-18 of rat Tra2β essentially as described for a different anti-peptide antibody (Matsuo et al., 1995).

In Vitro Assays

SELEX with in vitro phosphorylated His-tagged Tra2 proteins was performed essentially as described (Tacke and Manley, 1995). All other in vitro binding assays were performed as described previously (Tacke et al., 1997). Templates for IgM-based enhancer substrates were prepared by inserting the enhancer sequences A3 (Tacke and Manley, 1995) or B3 (Tacke et al., 1997) into the SalI site of pμMΔ (Watakabe et al., 1993), kindly provided by A. Mayeda, and transcribed by SP6 RNA polymerase after linearization with XbaI (A3) or AluI (B3). Competitor RNAs were prepared by T7 transcription of previously described template plasmids (Tacke and Manley, 1995; Tacke et al., 1997) linearized with XbaI and quantitated by trace-labeling with (α^{32}P)-GTP in parallel transcription reactions, assuming that transcription efficiency was the same in the presence and absence of the labeled nucleotide. In vitro splicing was performed essentially as described (Tacke and Manley, 1995). For competition assays, competitor RNA was added together with the substrate to the splicing reaction. To relieve competition, Tra2 or SR proteins were added last.

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